Antigen Identification for Antibodies against Vaccinia Virus Utilizing Nucleic Acid Programmable Protein Arrays (NAPPA) Technology

E. Randal Hofmann, Ph.D.[2], Jennifer Viloria[3], Andrea Throop[3], Alena Calm[1], Catherine Seiler[3], Ji Qiu, Ph.D.[3], Josh Labaer, Ph.D.[3]

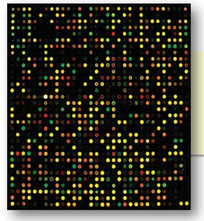
(1) US Army Edgewood Chemical Biological Center, (2) Excel, Inc., (3) Biodesign Institute, Arizona State University

Abstract

The production of antibodies to biowarfare agents generally employs the immunization of animals with inactivated whole organisms. While this process is very effective for antibody production, the immuno-stimulating antigens used in this technique have generally remained undefined. This creates a vulnerability to detection capabilities in cases where, whether through natural or engineered means, the epitope required for antibody detection is no longer expressed by the threat organism. Furthermore, having a complete understanding of the B cell epitopes in response to an infection agent can aid in the design of therapeutic antibodies and vaccines.

In this study, we use the highly flexible and customizable protein microarray technology called nucleic-acid programmable protein arrays (NAPPA) to identify the antigens of antibodies against the Orthopoxvirus, vaccinia (VACV). Using the cell-free microarrays, *in vitro* synthesis of target proteins from DNA templates printed directly on the array, allows polypeptides to be captured immediately after translation, thereby avoiding the usual risks of protein aggregation and instability. By reducing the direct manipulation of target polypeptides, thousands of polypeptides can be generated and tested simultaneously *in situ*, being produced just-in-time for experiments while circumventing the common problems of protein purification and stability. Proteins from an organism's entire genome can be immobilized on a single array to facilitate fast antibody recognition screening without using live agent.

To date, 197 open reading frames from VACV western reserve (WR) were cloned into the NAPPA expression plasmids (~90% of the virus genome). Arrays have been made and QC tests confirm ~98% of the genes are expressed. Up to 86 monoclonal or polyclonal antibodies or antisera from the Critical Reagents Program (CRP) and commercial sources can be screened using these arrays to identify immunodominant antigens of VACV. We have created a shared resource for the research community through the DNASU Plasmid Repository (https://dnasu.org) for use in customizable platforms.

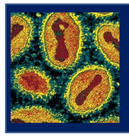


NAPPA reduces direct manipulation of target proteins, producing them just-in-time for the experiment and avoiding problems with protein purification and stability.

Thousands of proteins can be produced and tested simultaneously *in situ*.

Background

Antibodies in the CRP collection are made by immunizing animals with inactivated toxin, bacterial or viral antigens. In the case of bacterial and viral antigens, the entire microorganism is used to immunize the animal. This often produces a robust humoral immune response to immunodominant antigens on the surface of the microorganism. A major gap in the knowledge of these reagents is the identity of the target antigens for these antibodies among the hundreds of antigens on the surface of a microorganism. A high throughput technology platform to identify the antigen target of these antibodies would be highly desirable to the CRP.

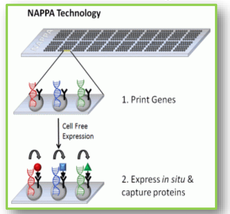


Plaguing human populations for thousands of years, worldwide deaths from the smallpox virus has been estimated at 300 million in the twentieth century alone. Smallpox, caused by an infection of *variola* virus, has an approximate 30% mortality rate and is primarily transmitted via inhalation and touch.

The NAPPA advantages over conventional methods:

- The printing of purified proteins or synthetic peptides is replaced with the **more reliable and less expensive process of printing DNA,** thereby avoiding the need to express, purify and store proteins.
- **The NAPPA method displays better than 95% of sequence-verified full-length genes**, including membrane proteins.
- **Display levels are more consistent from protein to protein**; 93% of display levels are within 2-fold of the mean. NAPPA arrays have only modest variation in protein expression from gene to gene (coefficient of variation = 24%). By having a more even display of proteins, data interpretation becomes more clear and simplified.
- Protein integrity is assured by using both mammalian expression machinery and chaperone proteins to synthesize and fold proteins. As proteins are very labile molecules, the advantage of the NAPPA method is the "just-in-time" production of fresh proteins on array, therefore avoiding potential denaturation during storage.

Nucleic Acid-Programmable Protein Array (NAPPA)



This protein microarray technology replaces the complicated process of spotting purified proteins with the easier process of plasmid DNA spotting.

Through the expression of multiple proteins on a single array we can test the function of multiple proteins simultaneously.

Transfer of protein encoding regions (open reading frames; ORFs) into specifically-tagged expression vectors

Methods

Construction of VACV WR NAPPA.
Genes encoded by VACV WR were first cloned into a Gateway pDONR vector and then transferred into the pANT7_cGST expression vector through one-step recombinational cloning. pANT7_cGST supports the expression of target genes with c-terminal GST fusion tags, thus allowing efficient capture *in situ* by anti-GST antibodies on our array platform. All constructs were end-sequence verified to ensure correct identities. VACV WR proteome arrays were constructed using a high-throughput preparation of high-quality plasmid DNA via the Biomek FX automated laboratory workstation. Purified DNA was printed onto aminosilane-coated glass slides using a Genetix Q-Array printer to produce VACV proteome protein arrays. On each array, each gene was printed in duplicate in a block with two blocks printed per array (allows for screening of two different antibodies on one array). Pico green was used to confirm successful and uniform printing of the DNA expression constructs. Arrays were incubated with HeLa cell extracts, as described below, and protein expression was confirmed using an anti-GST tag antibody.

Processing NAPPA Slides with Antibody.
Slides were blocked for one hour at room temperature, rinsed with Milli-Q water and dried with filtered compressed air. A mix of Hela cell lysate, accessory proteins, and reaction mix was then added to each slide after addition of a HybriWell gasket. Slides were incubated for 1.5 hours at 30ºC for protein expression and then transferred to 15ºC for GST-tag capture. The HybriWell was then removed and slides were rinsed twice with phosphate buffered saline (PBS) Tween. After three more washes, slides were blocked again with milk for 30 minutes at room temperature. Antibodies were prepared in PBS milk (5%) at dilutions ranging from 1:5 to 1:5000, depending on the antibody. The primary antibody solutions were added to the slides and incubated for one hour at room temperature. Slides were then washed with milk then secondary antibodies consisting of Alexa647 labeled goat anti-species antibodies were diluted 1:500 (12.5 µM) and incubated with the slides for one hour at room temperature with protection from light. Slides were washed and then scanned on a Tecan PowerScanner at various gain settings for optimal resolution and signal to noise. The fluorescent signal intensity was quantified using Array-Pro Analyzer (Media Cybernetics, Bethesda, MD).

Antibody ID	Description	Specificity	Reactive Antigens
ab35219	Rabbit polyclonal	n/a	D8L (VACWR113) A10L (VACWR129) A26L (VACWR149) H3L (VACWR101)
ab93829	Rabbit polyclonal	G9R (VACWR087)	None found
ab117453	Rabbit polyclonal	n/a	B2R (VACWR184) D12L (VACWR117) I1L (VACWR070)
ab21098	Mouse monoclonal	n/a	None found
ab156854	Mouse monoclonal	A27L (VACWR150)	A27L maxiprep control, A27L (VACWR150); from miniprep
AB-R-VACC	Rabbit Polyclonal	n/a	D8L (VACWR113) A10L (VACWR129) A26L (VACWR149) H3L (VACWR101) I1L (VACWR070)
AB-VACC-MAB1	Mouse monoclonal		I1L (VACWR070)
AB-VACC-MAB2	Mouse monoclonal	unknown	None found
AB-VACC-MAB3	Mouse monoclonal	ND	ND
AB-VACC-MAB4	Mouse monoclonal	L1R	None found
AB-VACC-MAB5	Mouse monoclonal	unknown	None found
AB-VACC-MAB6	Mouse monoclonal	unknown	I1L (VACWR070)

Table 1. Summary of results from antibody testing on VACV NAPPA.

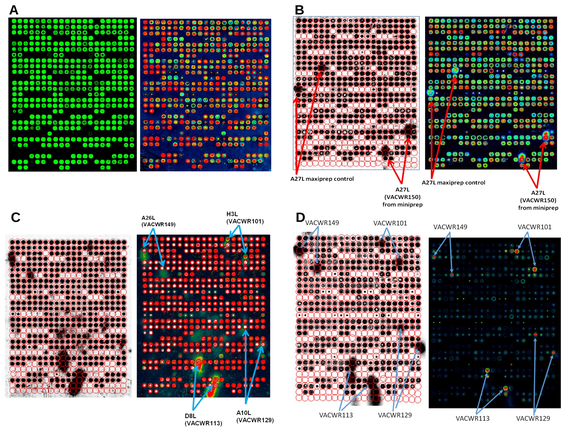


Figure 1. Quality control analysis and characterization of the VACV NAPPA. (A) Pico green (left) and anti-GST evaluation of VACV NAPPA. (B) Evaluation of ab156854 mouse anti-A27L monoclonal antibody (1:5). (C) Evaluation of ab35219 rabbit anti-VACV (1:200). (D) Evaluation of AB-R-VACC rabbit anti-VACV (1:500)

Protein	Function
G9R	poxvirus myristoylprotein; Entry-fusion complex protein
A27L	IMV surface protein; fusion protein
D8L	IMV membrane protein
I1L	putative DNA-binding virion core protein
A10L	precursor p4a of core protein 4a
A26L	cowpox A-type inclusion protein
H3L	IMV heparin binding surface protein
B2R	hypothetical protein
D12L	small subunit of mRNA capping enzyme

Table 2. Summary of VACV antigens and their function.

Results

To produce VACV NAPPA arrays, VACV genes were cloned into an expression vector that supports *in vitro* protein expression. A collection of 197 VACV NAPPA expression clones was produced, which represents 90% coverage of ~220 genes encoded by the VACV WR genome, as annotated by the Poxvirus Bioinformatics Resource Center (http://poxvirus.org/). Genes were first cloned into a Gateway pDONR vector and then transferred into the pANT7_cGST expression vector through one-step recombinational cloning. pANT7_cGST supports the expression of target genes with c-terminal GST fusion tags, thus allowing efficient capture *in situ* by anti-GST antibodies on our array platform. After end-sequence verification, we prepared DNA for all constructs using our automated DNA factory. On each array, each gene was printed in duplicate in a block with two blocks printed per array (allows for screening of 2 different antibodies on one array). Pico green was used to confirm quality of printing of the DNA expression constructs. Arrays were incubated with HeLa cell extracts as described above and protein expression was confirmed using an anti-GST tag antibody confirming 98% of genes expressed over the raw intensity threshold (Figure 1A).

Six commercially purchased antibodies (abcam) were used to probe the VACV NAPPA arrays for reactive antigens (Table 1). The monoclonal antibody ab156854 is reported to be specific to the VACV protein A27L. Figure 1 shows that all four spots for DNA expressions plasmids corresponding to the A27L ORF have noticeably higher signal than background signal on the array.

A control antibody (ab93829) raised against a recombinant fragment of G9R was also used but no noticeable signal was detected over background (data not shown). Two other monoclonal antibodies (ab21098, ab48569) of unknown specificity also failed to detect any NAPPA produced proteins (data not shown). Two polyclonal antibodies showed reactivity to multiple antigens on the VACV NAPPA arrays (Figure 1C and Table 1). There was no overlap in the antigens reactive to these two polyclonal antibodies.

Antibodies from the CRP were used to probe the slides to determine the antigens recognized by these antibodies. A rabbit polyclonal used in the study identified the same four proteins one of the abcam polyclonal antibodies recognized (Figure 1D and Table 1). It may also recognize an additional protein, I1L. This protein was also detected by two of the monoclonal antibodies tested from the CRP (Table 1).

Discussion

Preliminary results suggest that the proteins produced by the NAPPA technology are presenting viral epitopes that are important for driving the humoral immune response as demonstrated by the positive reaction with the polyclonal antibodies. Several proteins identified, including D8L, H3L and A27L, are part of the intracellular mature virion (IMV) surface and known to be important for neutralization of the virus[1]. We tested three monoclonal antibodies claimed to be specific to the protein A27L by abcam. Only one of these monoclonal antibodies reacted with A27L while the other two did not react at all. A polyclonal antibody raised against a N-terminal peptide from G9R and a monoclonal against the protein L1R did not react to their respective proteins on the array. This suggests that some epitopes may not be presented on these arrays although we can't rule out the possibility that the antibodies used in these cases are not strong binders or of high quality. L1R is a particularly challenging antigen as it is known that the neutralizing epitope for the antibody used in this experiment is complex and dependent on disulfide bond formation. Cell-free protein synthesis is not the most efficient at producing disulfide bonds and recent papers suggest using systems enriched with microsomal vesicles derived from the endoplasmic reticulum[2]. Our results indicate that the VACV NAPPA arrays are a good tool for probing for immunodominant antigens.

References

1. Moss, B., *Smallpox vaccines: targets of protective immunity.* Immunol Rev. 2011 Jan: 239(1): 8-26.
2. Buntru M, Vogel S., Stoff K., Spiegel H., Schillberg A. *A versatile coupled cell-free transcription-translation system based on tobacco BY-2 cell lysates.* Biotechnol Bioeng, 2015 May;112(5):867-78. doi: 10.1002/bit.25502. Epub 2015 Jan 16.

Acknowledgements: The authors thank the Defense Threat Reduction Agency/Joint Science and Technology Office for their assistance and funding of this work. The views expressed in this presentation are those of the authors and do not necessarily reflect official policy or the position of the Department of Defense or the U.S. Government.